

Award Number: **W81XWH-11-1-0704**

TITLE: Chemotherapy necessitates increased immune control of HHVs: A cause of persistent inflammation enabling protracted fatigue in breast cancer survivors

PRINCIPAL INVESTIGATOR: Jessica E Thaxton, PhD

CONTRACTING ORGANIZATION: Medical University of South Carolina
Charleston, SC 29425

REPORT DATE: June 2015

TYPE OF REPORT: Final Summary

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

REPORT DOCUMENTATION PAGEForm Approved
OMB No. 0704-0188

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Department of Defense, Washington Headquarters Services, Directorate for Information Operations and Reports (0704-0188), 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302. Respondents should be aware that notwithstanding any other provision of law, no person shall be subject to any penalty for failing to comply with a collection of information if it does not display a currently valid OMB control number. PLEASE DO NOT RETURN YOUR FORM TO THE ABOVE ADDRESS.

1. REPORT DATE June 2015		2. REPORT TYPE Final Summary		3. DATES COVERED 15 Sept 2011 – 30 April 2015	
4. TITLE AND SUBTITLE Chemotherapy necessitates increased immune control of HHVs: A cause of persistent inflammation enabling protracted fatigue in breast cancer survivors				5a. CONTRACT NUMBER 016957-002	
				5b. GRANT NUMBER W81XWH-11-1-0704	
				5c. PROGRAM ELEMENT NUMBER	
6. AUTHOR(S) Jessica Thaxton, PhD, MsCR E-Mail: Thaxton@musc.edu				5d. PROJECT NUMBER	
				5e. TASK NUMBER	
				5f. WORK UNIT NUMBER	
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) Medical University of South Carolina 179 Ashley Avenue Charleston SC 29425				8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012				10. SPONSOR/MONITOR'S ACRONYM(S)	
				11. SPONSOR/MONITOR'S REPORT NUMBER(S)	
13. SUPPLEMENTARY NOTES					
14. ABSTRACT We hypothesized that chemotherapy would induce a shift in control of chronic human herpes viruses (HHVs) in breast cancer patients and that this alteration may drive protracted cancer treatment related fatigue (CTRF) on account of enhanced inflammatory cytokine production. We examined three cohorts of patients in order to test our hypothesis. Cohort 1 consisted of breast cancer patients actively undergoing chemotherapy (n=14). Cohort 2 was comprised of breast cancer patients who had previously undergone chemotherapy and continued to present with active disease (n=20). Cohort 3 consisted of breast cancer survivors, previously treated with chemotherapy, who were disease free for at least two years (n=97). We found that EBV/CMV double positive status was predictive of significantly elevated levels of sera INF- γ in Cohort 1 and Cohort 2. Analysis of CD8+ T cells from PBMC showed that immuno-dominant CMV epitope IE1 drove elevated IFN- γ levels in breast cancer patients who were undergoing chemotherapy compared to all other viral epitopes tested. IFN- γ derived from IE1-specific CD8+ T cells was predictive of serum CRP levels, albeit a negative correlation. In subjects with active disease and previously treated with chemotherapy, EBV+/CMV+ status predicted a significant and positive association between serum IFN- γ and serum CRP levels. Finally, among breast cancer survivors, EBV+/CMV+ patients showed a significant and positive correlation between CTRF score and serum CRP, and this relationship did not exist in EBV+ alone subjects. Taken together our data suggest that, in association with chemotherapy, CMV drives an enhanced inflammatory milieu in breast cancer patients and survivors marked early by IFN- γ produced by IE1 specific CD8+T cells and marked later by elevated levels of CRP. Further, the aforementioned parameters may be strongly predictive of protracted CTRF in breast cancer patients and survivors.					
16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT UU	18. NUMBER OF PAGES 18	19a. NAME OF RESPONSIBLE PERSON USAMRMC
a. REPORT U	b. ABSTRACT U	c. THIS PAGE U			19b. TELEPHONE NUMBER (include area code)

Table of Contents

	Page
Introduction.....	4
Body.....	5
Key Research Accomplishments.....	13
Reportable Outcomes.....	14
Conclusion.....	15
Statement of Work.....	16
References.....	18

INTRODUCTION:

We hypothesized that chemotherapy would induce a shift in control of chronic human herpes viruses (HHVs) in breast cancer patients and that this alteration may drive protracted cancer treatment related fatigue (CTRF) on account of enhanced inflammatory cytokine production. Over three subject groups assessed we consistently found that CMV+/EBV+ subjects showed significant differences in inflammatory status, virus-specific T cell responses, and CTRF scores compared to EBV+ alone subjects. Other combinations of HHVs were not significantly different from one another. Thus, we focused our study on CMV+ versus CMV- subjects as we found CMV to be the strongest driver of HHV-associated inflammatory immune changes.

We initially proposed to follow subjects over time and assess parameters of HHV status, serum cytokines, virus-specific T cell responses, and CTRF scores. Due to difficulties with longitudinal sample collection discussed in previous annual reports we adapted our strategy to look at the aforementioned parameters in three subject cohorts (Table 1). Cohort 1 was comprised of n=14 female breast cancer patients actively undergoing chemotherapy. Cohort 2 was comprised of n=20 female breast cancer patients with active disease, previously treated with chemotherapy, but not actively undergoing treatment. Cohort 3 was comprised of n=108 breast cancer survivors, survived 2-10 years, with no signs of active disease. In all cohorts we surveyed viral status for HHVs HSV-1, VZV, EBV, and CMV. We assessed the following serum cytokine levels in all cohorts: IL-10, neopterin, TNF- α , IL-6, IFN- γ , and CRP. In cohort 1 and 2 we assessed virus-specific T cell responses from PBMC. Cohort 2 data were collected from previously banked frozen PBMC and data from frozen samples proved unreliable given a lack of positive cytokine signal from positive control treated cells. Thus, for cohort 1 we developed a novel 7day *in vitro* T cell culture protocol for PBMC taken directly from subjects and our results were reliable given positive and negative controls. PBMC from cohort 3 were not available to us for assessment of virus-specific T cell responses in breast cancer survivors. Fatigue scores from cohort 1 were not collected given that subjects were in active chemotherapy and thus fatigue reported would be considered acute CTRF, not protracted. Fatigue scores from cohort 2 were not available to us given that this was a retrospective cohort. Fatigue scores from cohort 3 were available to us and were based on the PROMIS fatigue survey (NIH) where 5 was the least level of fatigue and 40 was the greatest level of fatigue a patient could report.

Table 1. Characteristics and Parameters of 3 Cohorts Assessed

Cohort	N	Disease Status	Chemotherapy Status	Viral Status	Virus-Specific T cell responses	Serum Cytokines	Fatigue Score
Cohort 1	14	Active	Active	X	X	X	N/A
Cohort 2	20	Active	Previously received	X	X Analysis N/A	X	N/A
Cohort 3	108	Not active 2-10yr survivors	Previously received	X	N/A	X	X

The body of this report details the significant findings in each cohort of subjects assessed. We are able to tie together data collected and analyzed in each phase of disease and recovery to show consistent trends among cohorts. Our data suggests that acute disease coupled to active chemotherapy leads to significantly higher levels of serum IFN- γ found in EBV+/CMV+ subjects as compared to EBV+ alone. Virus-specific T cell responses, from PBMC, produced high levels of IFN- γ from the CD8+ T cell compartment and CMV+ responses were dominant over EBV+

responses. In keeping with the significant findings from cohort 1, cohort 2 subjects who were EBV+/CMV+ had significantly higher levels of serum IFN- γ and serum CRP compared to EBV+ only subjects. Further, serum IFN- γ levels in EBV+/CMV+ subjects were positively correlated with CRP levels. Finally, assessment of cohort 3 showed a trend for EBV+/CMV+ subjects to have higher serum CRP compared to EBV+ alone subjects. Strikingly, CRP levels showed a significant and positive association with CTRF scores in subjects that were EBV/CMV double positive, not EBV+ alone subjects. Taken together our data suggest that CMV driven T-cell mediated IFN- γ production leads to increased inflammatory milieu in breast cancer patients and survivors that is marked by higher long-term levels of CRP that correlate with enhanced CTRF.

BODY:

Hypothesis: This work hypothesizes that chemotherapy can permanently alter the balance between the immune system and chronic herpesvirus infections and the resultant increase in inflammatory cytokines exacerbates CTRF.

Specific Aim 1: To determine whether the number of HHV infections and/or the type of HHV infection carried by an individual contributes to protracted fatigue in BC survivors.

Specific Aim 2: To monitor fatigue levels, HHV infections, and HHV-specific immunity in BC patients during chemotherapy to assess the impact of therapy on immune control of HHVs and CTRF outcomes.

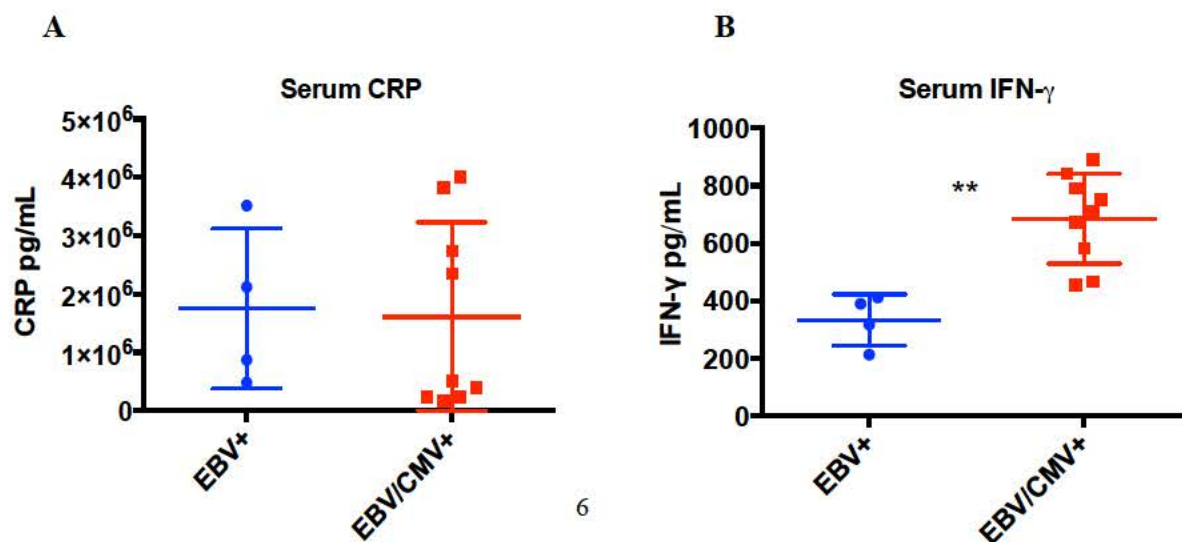
Cohort 1: Description & Significant Findings

We collected n=14 subject samples from females that were actively undergoing chemotherapy for breast cancer. Samples collected were PBMC and serum. We did not collect fatigue data from subjects as patients were in active treatment and fatigue reports would have been specific to acute cancer treatment related fatigue, not protracted. We assessed viral status by ELISA for the four major human herpes viruses and CMV/EBV status is shown in Table 2. We assessed serum for inflammatory cytokines IL-10, neopterin, TNF- α , IL-6, IFN- γ , and CRP and the latter two values are reported in Table 2. Finally, we assessed virus-specific CD8+ T cell responses through peptide re-stimulation of immuno-dominant antigenic-epitopes for both CMV (pp65 & IE1) and EBV (BZLF1 & EBNA). Briefly, we first froze PBMC and stored them for peptide re-stimulation at a later time point. Using this method, analysis of samples 101 and 102 post-thaw proved an inadequate method for reliable results as the positive control for T-cell specific cytokine production, PMA/Ionomycin stimulation, was not significantly positive for INF- γ production above viral-peptide treated controls. Thus, we developed a T-cell culture method from PBMC similar to that employed to culture tumor infiltrating lymphocytes (TIL) from tumor microenvironment. We utilized h-T cell media supplemented with fetal calf serum and 6000U of IL-2 to culture PBMC for 7 days. This method proved reliable to culture and grow CD8+ T cells from human PBMC. CD8+ T cells proliferated in culture and we were able to reliably measure IFN- γ responses at day 7 from the cultures after 4-6 hours of peptide re-stimulation supplemented with golgi block Brefeldin A. Positive controls were PMA/Ionomycin treated wells and negative controls received no stimulation.

Table 2. Cohort 1 Viral Status and Inflammatory Parameters

Patient	CRP pg/mL	IFN- γ pg/mL	CMV	EBV	Total IFN	CMV-pp65	CMV-IE1	EBV-BZLF	EBV-EBNA
101	482000	412	0	1	NA	NA	NA	NA	NA
102	2120000.472	214	0	1	NA	NA	NA	NA	NA
103	168823.529	842	1	1	6.8	0	1	3.5	2.3
104	4009411.765	672	1	1	1.86	0	0	0	1.86
105	2347058.824	456	1	1	47.36	14.1	1.76	15.2	16.3
106	398235.294	468	1	1	7.58	1.18	4.7	1.7	0
107	2371176.471	512	1	0	1.26	0.43	0.83	NA	NA
108	225294.117	892	1	1	6.27	0.34	5.93	0	0
109	2737058.824	752	1	1	9.3	0	0	8.9	0.4
110	232352.941	790	1	1	44.85	0.45	15.5	16.5	12.4
111	3815882.353	712	1	1	3.85	0.28	0.78	2.44	0.35
112	870000	318	0	1	0	NA	NA	0	0
113	509411.764	582	1	1	9.71	2	3.23	2.95	1.53
114	3514705.882	390	0	1	1.33	NA	NA	0	1.33

Of n=14 subjects collected n=4 were EBV+ alone, one subject was CMV+ alone, and n=9 subjects carried both EBV and CMV (0=no, 1=yes) (Table 2). CRP values were an important predictor of CTRF in cohort 3 and we assessed CRP levels from serum via ELISA in this cohort of subjects. We found that CRP values were 10 to 100 fold higher than CRP values from cohort 2 or cohort 3 and we attributed this to the fact that subjects were actively undergoing chemotherapy – a time of vast inflammatory response. Not surprisingly, at this early treatment time point there were no detectable differences between EBV+ alone and EBV+/CMV+ subject groups (Figure 1A). In contrast, serum IFN- γ was significantly elevated in EBV+/CMV+ subjects as compared to the EBV+ alone group with means of 685.1 ± 51.91 and 333.5 ± 44.60 , respectively, and a p value of $p=0.0016$ (Figure 1B).

Figure 1. EBV+/CMV+ breast cancer patients undergoing chemotherapy have significantly elevated serum IFN- γ 

Given high levels of IFN- γ in sera of EBV+/CMV+ subjects we wished to examine IFN- γ derived from virus-specific T cell responses to determine whether CMV-specific T cell responses may predominate over EBV+ responses. Of n=14 subjects, we were able to reliably assess virus-specific T cell responses from n=12 subjects. Of n=12 subjects, n=1 subject produced no virus-specific T cell responses. Figure 2 depicts the remaining n=11 subjects and their immuno-dominant virus-specific CD8+ T cell responses measured by IFN- γ production as assessed by flow cytometry. Of n=6 subjects surveyed that were EBV+/CMV+, IE1 responses produced the most CD8-derived IFN- γ . EBV-derived BZLF1 was secondarily dominant to IE1.

Figure 2. Individual breast cancer patient CMV and EBV virus-specific CD8+ T cell IFN- γ responses

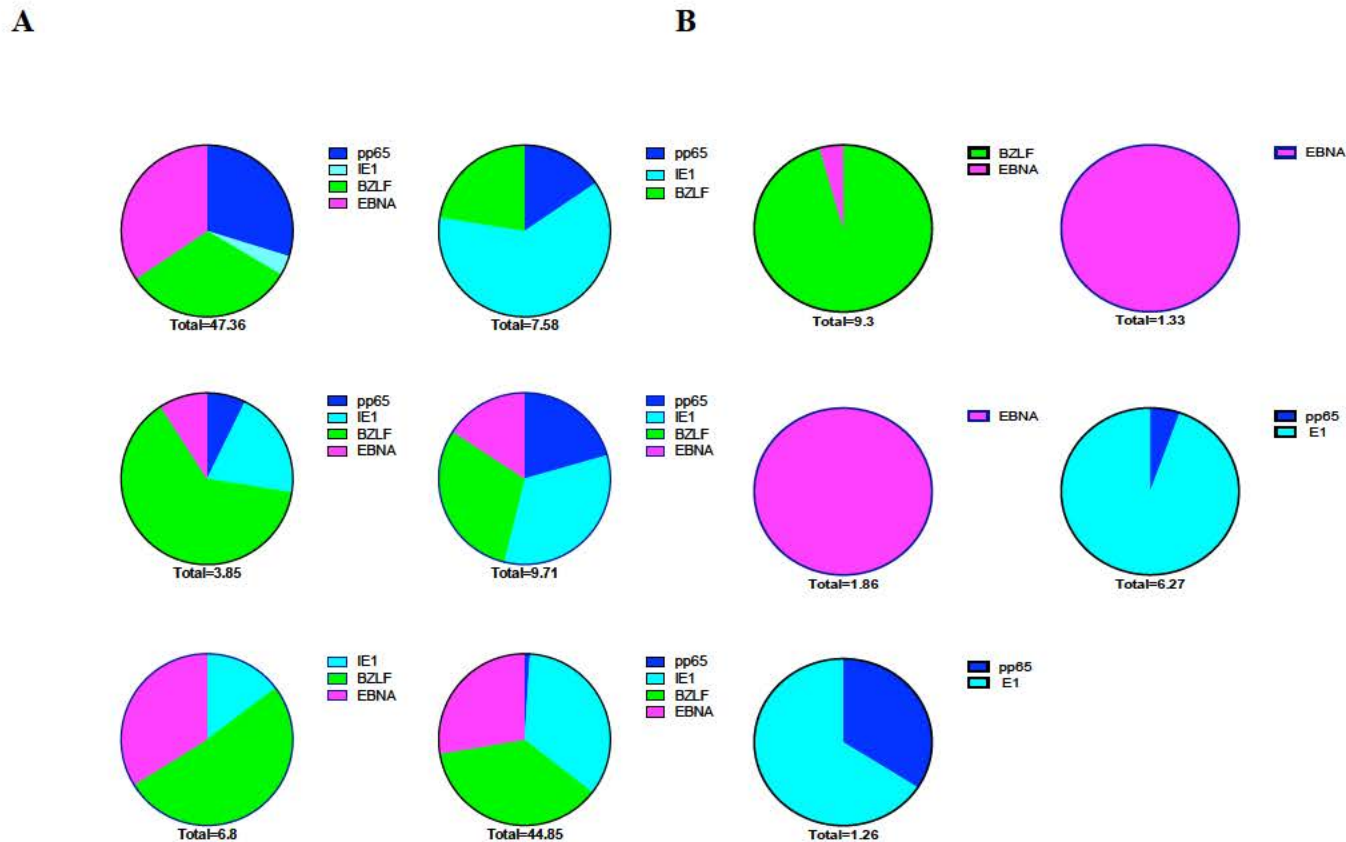
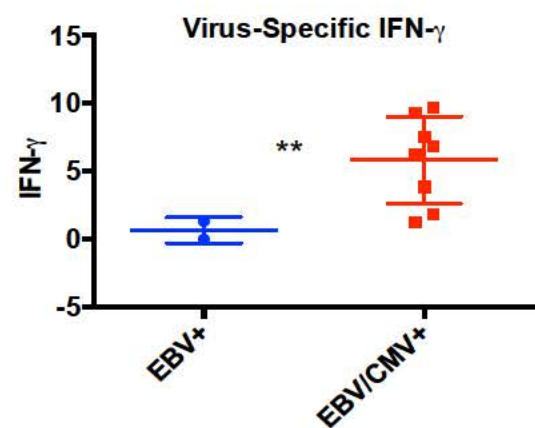


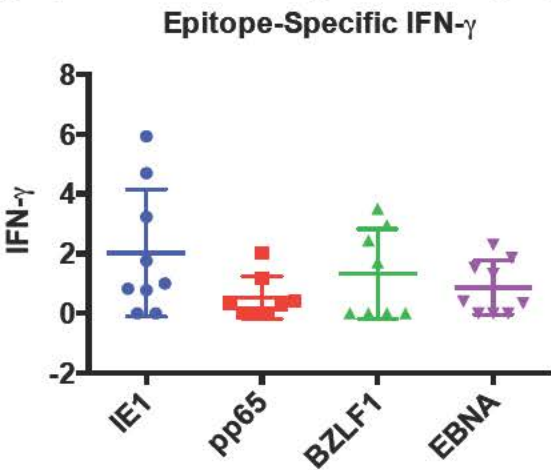
Figure 3. Total virus-specific CD8+ T cell IFN- γ production stratified by viral infection in breast cancer patients undergoing chemotherapy



On the whole, subjects who were EBV+/CMV+ produced more virus-specific T cell derived IFN- γ than subjects who were EBV+ alone, 0.6650 ± 0.6650 and 5.829 ± 1.133 , respectively with a p-value of $p=0.0058$ (Figure 3). We next looked at IFN- γ as a product of viral antigenic epitopes to quantify whether one particular epitope produced the greatest inflammatory response above others. Indeed, CMV epitope IE1

produced an immuno-dominant response compared to CMV epitope pp65 and EBV epitopes BZLF1 and EBNA (Figure 4). Though not significant in this small pool of data, if our n were expanded we predict that IE1 will be significantly more indicative of CD8+ T cell-driven inflammatory capacity as compared to other viral antigenic epitopes.

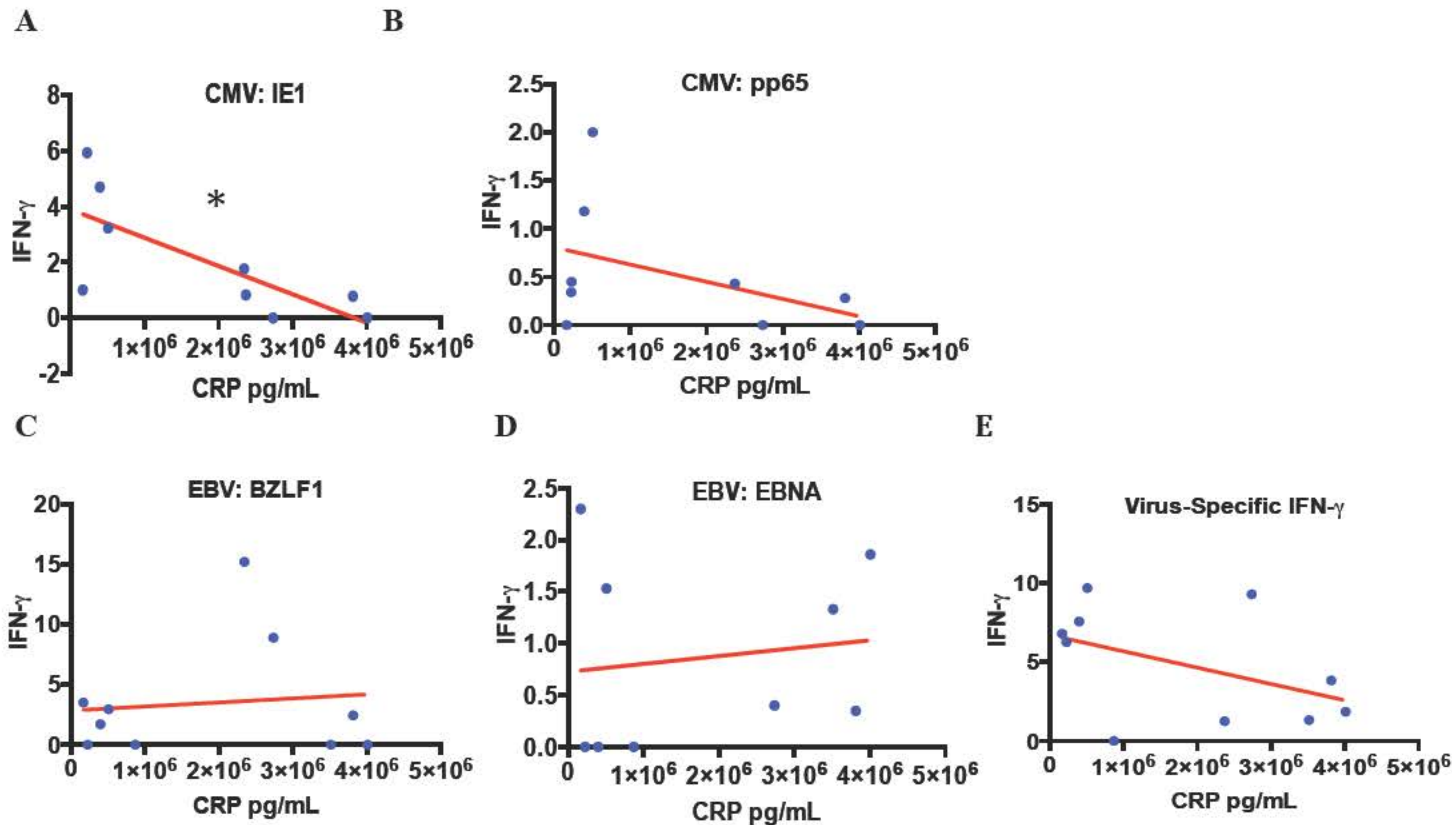
Figure 4. Virus-specific CD8+ T cell IFN- γ production stratified by immuno-dominant viral epitopes in breast cancer patients undergoing chemotherapy



Given that cohort 2 and cohort 3 showed significantly elevated CRP levels in the EBV+/CMV+ subject groups compared to EBV+ alone groups we wished to determine whether a relationship existed between viral epitope-specific IFN- γ and CRP production. We performed linear regression analysis between IFN- γ produced in response to virus-specific peptide restimulation and serum CRP levels. We found a significant and negative correlation between IFN- γ and CRP in the IE1 antigenic epitope group ($p=0.0227$, $r=-0.7397$) (Figure 5A). Similarly, CMV antigenic epitope pp65 showed a negative correlation between IFN- γ and

CRP, though this correlation was not significant ($p=0.2405$) (Figure 5B). As Figures 5C and D demonstrate the relationship between IFN- γ and CRP for EBV-specific major antigenic epitopes BZLF1 and EBNA had a slightly positive association but were not significant ($p=0.7669$, $p=0.7160$, respectively). In keeping with Figures 1B, 3, and 4, total virus-specific IFN- γ , a composite variable of total IFN- γ produced by both CMV and EBV major antigenic epitopes upon peptide restimulation, showed a negative association between IFN- γ and CRP that was significant when $\alpha=0.2$, confidence interval=80% ($p=0.1816$, $r=-0.4595$) (Figure 5D).

Figure 5. Correlation of viral epitope-specific CD8+ T cell IFN- γ and serum CRP levels in breast cancer patients undergoing chemotherapy



Significant findings from cohort 1 show elevated sera IFN- γ in subjects double positive for EBV/CMV compared to EBV positive subjects alone (Figure 1B). We next found that virus-specific CD8⁺ T cell-derived IFN- γ was greater in the double positive group as well and these data indicate that IFN- γ from CMV-specific CD8⁺ T cells contributed to enhanced inflammatory conditions in EBV/CMV double positive subjects (Figure 3). We further confirmed the contribution of CMV-mediated inflammation through assessment of IFN- γ produced by specific viral major antigenic epitopes. We found IE1-specific CD8⁺ T cells produced the highest levels of IFN- γ compared to CMV epitope pp65 or EBV epitopes BZLF1 and EBNA (Figure 4). Finally, IE1-specific CD8⁺ T cells showed a significant yet negative correlation between IFN- γ produced and patient-specific serum CRP levels (Figure 5A). This trend also approached significance when total viral CD8⁺ T cell-derived IFN- γ was assessed (Figure 5E).

Cohort 2: Description & Significant Findings

Elevated CRP has been reported to correlate with CTRF and CRP has been shown to be an inflammatory mediator in CMV infection ^{1,2}. To date a link between CMV-driven inflammation correlated to enhanced levels of CRP in breast cancer patients that have undergone chemotherapy has not been established. Further, the relationship between CMV, chemotherapy, CRP, and protracted CTRF has not been explored. We utilized patient data from our second cohort of patients to expound upon the relationship between CMV serostatus, IFN- γ -derived inflammation, and CRP levels in serum.

We wished to determine whether we could detect significant associations between EBV/CMV double positive status and serum levels of IFN- γ or CRP in breast cancer patients previously treated with chemotherapy when compared to EBV positive alone subjects. We obtained n=20 banked breast cancer patient samples, serum and plasma, from the bio-repository at MUSC. At the time of sample collection all breast cancer patients had previously undergone chemotherapy but were not in active treatment. Table 1 depicts the de-identified patient samples tested. Histological and stage data demonstrate that subjects ranged in breast cancer type, stage, and metastatic activity (Table 3).

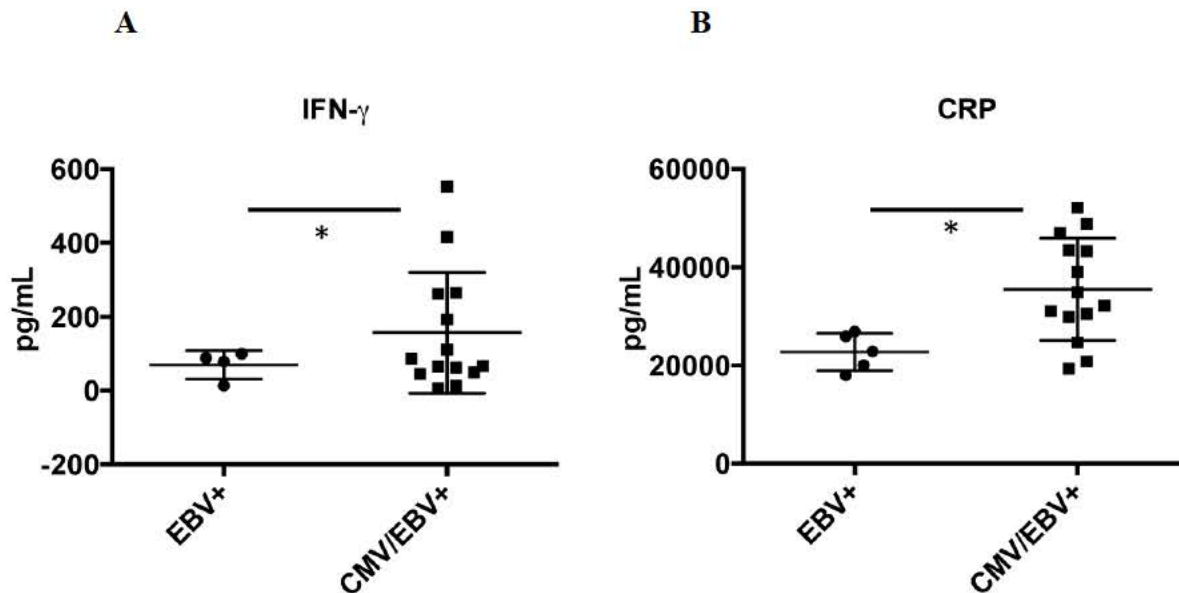
Table 3. Cohort 2: Breast cancer subject samples by histological type and stage.

Histology	Stage
invasive ductal carcinoma	T1cNO(ITC)Mx
lobular carcinoma	T1bN1Mx
lobular carcinoma	T2NxMx
invasive ductal carcinoma	T1aN1a
invasive ductal carcinoma	T1bN1a
invasive ductal carcinoma	T1cN1
invasive ductal carcinoma	T2Nx
invasive ductal carcinoma	T1bNO
invasive ductal carcinoma	
invasive ductal carcinoma	T3N2a
invasive ductal carcinoma	T1cNO
invasive ductal carcinoma	T1cN1a

invasive ductal carcinoma	T1bNx
invasive ductal carcinoma	T1bNO
ductal carcinoma	T1cN1a
invasive ductal carcinoma	T1bN1a
focal residual ductal carcinoma	
invasive lobular carcinoma	T1cNOMx
invasive ductal carcinoma	T1aNO
invasive ductal carcinoma	T1aNOMx

We first tested subject serum for CMV and EBV sero-status. Of n=20 subjects n=6 were positive for EBV alone and n=14 were double positive for EBV and CMV. IFN- γ and CRP are direct products of virus-specific T cell activation, thus we undertook IFN- γ and CRP ELISAs on subject serum as an indirect measure of virus-specific T cell activity.^{3,4} We assessed IFN- γ between viral groups and found mean values of 69.82 ± 19.27 pg/mL for the EBV+ group and 156.9 ± 43.72 pg/mL for the double positive virus group (Figure 6A). Two tailed t-test analysis with Welch's correlation of these means proved significant, $p=0.0359$. Further, our assessment of CRP showed EBV+ subjects had a mean value of 22767 ± 1687 pg/mL and CMV/EBV double positive subjects had a mean value of 35511 ± 2775 , pg/mL (Figure 6B). Two tailed t-test between the two virus groups and CRP was significant, $p=0.0011$. Taken together, these data show that CMV/EBV double positive viral status is significantly associated with enhanced CRP and IFN- γ levels in serum of active disease breast cancer patients that have previously undergoing chemotherapy.

Figure 6. Viral status is significantly associated with enhanced IFN- γ and CRP levels in breast cancer patients who have previously undergone chemotherapy



We next aimed to determine whether CRP and IFN- γ levels were associated with one another on a per patient basis in the EBV+ group or in the CMV/EBV double positive group. We performed linear regression analysis on the four samples from our cohort of breast cancer patients that were EBV+ only and found no significant correlation between CRP and IFN- γ ($p=0.2549$). Strikingly, in the double positive virus group, a linear regression of CRP and IFN- γ levels of each subject within this groups showed a positive and significant association where $p=0.0432$ (Figure 7). These data

demonstrate that EBV+/CMV+ breast cancer patients, who have previously undergone chemotherapy, have elevated levels of sera IFN- γ and this is predictive of elevated CRP levels as well.

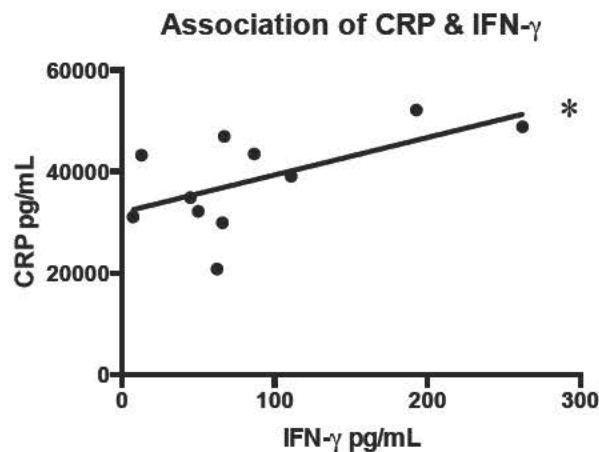


Figure 7. CRP and IFN- γ levels are positively and significantly associated in EBV/CMV+ breast cancer patients who have previously undergoing chemotherapy.

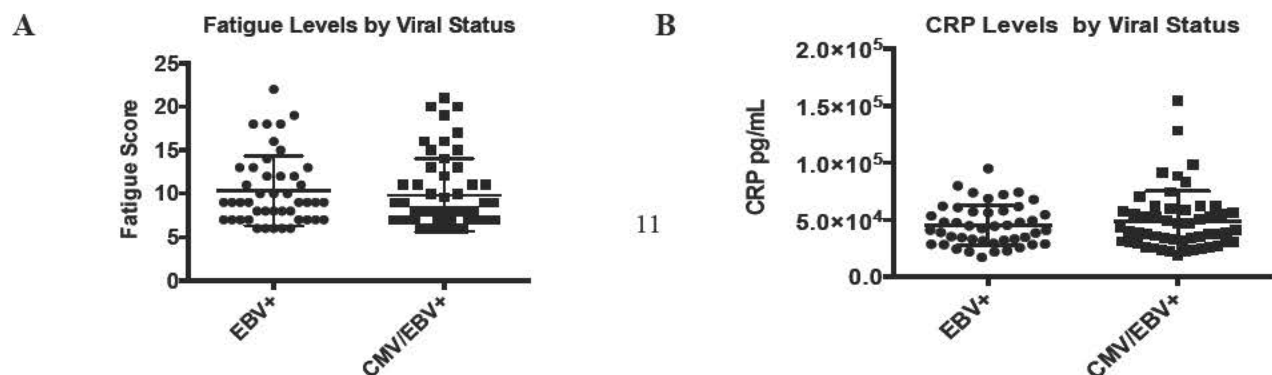
From data analysis of cohort 1 and cohort 2 a picture emerges of elevated inflammation in EBV/CMV double positive subjects compared to EBV positive subjects. Cohort 2 data confirm elevated IFN- γ in sera of EBV+/CMV+ subjects compared to EBV+ subjects and begin to suggest a correlation in elevated CRP levels as well that is

associated with double positive viral status (Figure 6). Importantly, serum CRP associations in stratified viral groups were not detectable in cohort 1. CRP levels were 10-100 fold greater in subjects actively undergoing chemotherapy. In cohort 2 CRP levels were dramatically decreased from those found in cohort 1 and this is likely due to the acute inflammation associated with active chemotherapy due to rapid cell death. Thus, the association of CRP and IFN- γ , specific to viral status, may be unmasked after subjects have been given time to recover from initial stages of chemotherapy (Figure 7).

Cohort 3: Description & Significant Findings

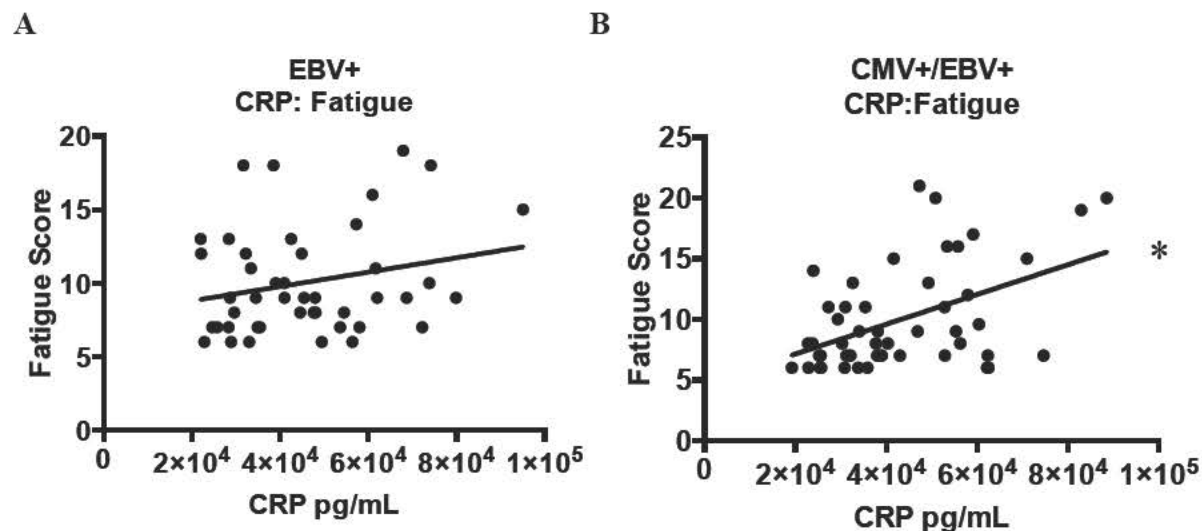
We wished to determine whether our significant findings from cohort 1 and cohort 2 were transferable to long-term breast cancer survivors and associated with CTRF. From cohort 3 we included 97 subjects to assess associations among viral status, serum cytokine levels, and fatigue scores. All subjects were breast cancer survivors who had previously received chemotherapy and who were disease free for at least 2 years. Survivors ranged in survival time from 2 to 10 years. Of 97 subjects assessed, 45 subjects were positive for EBV alone and 53 were positive for both EBV and CMV. Fatigue scores were a result of the PROMIS fatigue survey (NIH) where 5 was the least level of fatigue and 40 was the greatest level of fatigue a patient could report. We assessed whether combinations of viruses correlated with fatigue score or inflammatory cytokine production. We found that a detectable trend between CMV/EBV double positive virus status and fatigue score did not exist in breast cancer survivors (Figure 8A). However, we found that a trend existed where CMV/EBV double positive survivors showed higher levels of serum CRP compared to EBV single positive subjects (Figure 8B).

Figure 8. EBV/CMV status is not significantly associated with CTRF score or CRP levels in breast cancer survivors



We reasoned that a detectable association might not exist in survivor populations between CRP and EBV/CMV viral status. Viral reaction and immune-shaping most likely occur over the course of chemotherapy and upon immune-reconstitution post treatment. Due to the trend of increased CRP levels in EBV+/CMV+ subjects coupled to recent reports that suggest a positive correlation between CRP and CTRF we assessed the correlation between CRP levels and reported CTRF in EBV+/CMV+ subjects^{5,6}. As Figure 9 demonstrates, linear regression analysis showed a significant correlation between high fatigue score and elevated serum levels of CRP in EBV/CMV double positive subjects ($p=0.0006$, $r=0.4755$, Figure 9B), but not EBV+ alone subjects ($p=0.1223$, $r=0.2364$, Figure 9A).

Figure 9. CRP serum levels predict CTRF score in EBV+/CMV+ breast cancer survivors only



Taken together these data show that elevated sera CRP levels are predictive of increased CTRF only in EBV+/CMV+ breast cancer survivors (Figure 9).

KEY RESEARCH ACCOMPLISHMENTS

- EBV+/CMV+ breast cancer patients actively undergoing chemotherapy have elevated sera IFN- γ compared to EBV+ subjects alone (**Figure 1B**)
- Combined virus-specific CD8+ T cell-derived IFN- γ is greater in EBV+/CMV+ than in EBV+ breast cancer patients actively undergoing chemotherapy (**Figure 3**)
- CMV IE1-specific CD8+ T cells produce the highest levels of IFN- γ compared to CMV epitope pp65 or EBV epitopes BZLF1 and EBNA (**Figure 4**)
- CMV IE1-specific CD8+ T cells show a significant and negative correlation between IFN- γ produced and patient-specific serum CRP levels (**Figure 5A**)
- EBV+/CMV+ breast cancer patients with active disease previously treated with chemotherapy have significantly elevated sera IFN- γ compared to EBV+ alone subjects (**Figure 6A**)
- EBV+/CMV+ breast cancer patients with active disease previously treated with chemotherapy have significantly elevated sera CRP compared to EBV+ alone subjects (**Figure 6B**)
- Sera IFN- γ is positively and significantly correlated with sera CRP in EBV+/CMV+ breast cancer patients with active disease previously treated with chemotherapy but not in EBV+ alone subjects (**Figure 7**)
- Sera CRP positively predicts CTRF levels in EBV+/CMV+ long-term breast cancer survivors but not EBV+ subjects (**Figure 9**)

REPORTABLE OUTCOMES:

Scientific

Obtainment and analysis of n=14 prospective breast cancer subject samples (Cohort 1)

Obtainment and analysis of n=20 retrospective breast cancer subject samples (Cohort 2)

Obtainment and analysis of n=97 retrospective breast cancer subject samples (Cohort 3)

Preparation of manuscript to report the aforementioned findings, submission December 2015

Career Development

July 2014: Receipt of Masters in Clinical Research: GPA 4.0

September 2015: Attainment of Tenure-Track Assistant Professorship (MUSC)

September 2015: Establishment of Independent Cancer Research Lab (MUSC)

July 2015: Receipt of K12 Paul Calabresi Career Development Award for Clinical Oncology (NIH)

CONCLUSION:

Data from our analysis of cohort 1 suggest that EBV+/CMV+ subjects have greater sera IFN- γ than EBV+ subjects, and CD8+ T cell IFN- γ -mediated control of CMV immuno-dominant antigenic epitope IE1 drives this enhanced inflammation (Figures 1B, 3, 4). In cohort 1 subjects CRP values were elevated 10-100 fold above levels found in patients who were not actively undergoing chemotherapy. We determined these high levels of CRP to be a bi-product of treatment itself given the vast levels of acute inflammation that occur due to acute treatment. It is interesting to note that IE1-specific CD8+ T cell-derived IFN- γ remained corollary to CRP levels, albeit a negative correlation (Figure 5). These results suggest that at this early time point in breast cancer patients, CMV contributes to immune-shaping and long-term alteration in inflammatory milieu.

From data analysis of cohort 1 and cohort 2 a picture emerges of elevated inflammation in EBV/CMV double positive subjects compared to EBV positive subjects. Cohort 2 data confirm elevated IFN- γ and CRP in sera of EBV+/CMV+ subjects compared to EBV+ subjects (Figure 6). Importantly, serum CRP associations in stratified viral groups were not detectable in cohort 1. However, a striking correlation existed where subjects who produced the highest levels of IFN- γ also showed the greatest values of sera CRP. This correlation was only significant in subjects who were EBV+/CMV+ (Figure 7). These data suggest that CMV infection is a key causative factor in enhanced inflammatory conditions in breast cancer patients previously treated with chemotherapy.

Subjects who had previously undergone chemotherapy, and were survived 2 or more years did not show significant differences in IFN- γ levels when stratified by viral status. It is likely that CMV re-activation, and subsequent virus-specific T cell activity, occurs close to the window of chemotherapy application. Thus, CMV-derived IFN- γ may not be detectable two years post disease. CRP levels remained elevated in EBV+/CMV+ subjects only, a direct indicator that CRP levels were elevated in direct correlation with CMV infection. Along these lines, high levels of sera CRP predicted patient reports of elevated CTRF, and this relationship only existed in breast cancer survivors infected with CMV (Figure 9). These data are arresting and directly support a link between CMV infection and enhanced CTRF in breast cancer patients and survivors.

Statement of Work:

September 2012-October 2012

- | | |
|---|--------------|
| 1. Submit IRB to MUSC for expedited review to use samples from OHSU | Accomplished |
| 2. Meet with MUSC research team to plan prospective patient sample collection | Accomplished |
| 3. Prepare and submit full review IRB to MUSC for human subjects use approval | Accomplished |

November 2012-February 2012

- | | |
|---|--------------|
| 4. Receive retrospective cohort samples from OHSU | Accomplished |
| 5. Perform HHV analysis on patient samples (VZV, EBV, CMV, HSV-1) to determine seropositive/negative status | Accomplished |
| 6. IP-10 flow based assay, neopterin assay sample analysis | Accomplished |

February 2013-July 2013

- | | |
|---|-------------------------|
| 7. Coordinate coded fatigue data with serology and inflammatory protein results | Accomplished |
| 8. Statistical consultation/analysis for fatigue score with HHV type or # | Accomplished |
| 9. If necessary include total 285 subject data for enhanced significance | Accomplished |
| 10. Preparation of data and production of manuscript 1 | No significant findings |
| 11. Patient recruitment and sample collection for SA2 begins | Accomplished |

August 2013-January 2014

- | | |
|--|--------------|
| 12. Assess patient recruitment rates and sample collection efficacy | Accomplished |
| 13. Meet with study team for SA2 to revise and/or insure study maintenance | Accomplished |
| 14. Patient recruitment and sample collection for SA2 continues | Accomplished |
| 15. HHV analysis of baseline samples for SA2 to determine sero-status | Accomplished |
| 16. PCR of CMV+/EBV+ for viral DNA in sero+ samples from 4 th cycle | Not feasible |
| 3-6 month follow up to determine if viral DNA is detectable | |
| 17. IP-10 flow cytometry based assay | Not feasible |

February 2014-May 2014

- | | |
|--|--------------|
| 18. Assess patient recruitment rates and sample collection efficacy | Accomplished |
| 19. Meet with study team for SA2 to revise and/or insure study maintenance | Accomplished |
| 20. Patient recruitment for SA2 is completed | Accomplished |

Alternative n=20 subject samples collected from biorepository

- | | |
|--|--------------|
| 21. HHV analysis of baseline samples for SA2 to determine sero-status | Accomplished |
| 22. PCR of CMV+/EBV+ for viral DNA in sero+ samples from 4 th cycle | Not feasible |
| 23. Coordinate patient fatigue data with HHV sero-status and inflammatory data | Accomplished |
| 24. Statistical analysis/consultation for significance between SA1 parameters and SA2 (long-term vs short-term fatigue associations) | Accomplished |
| 25. Preparation and submission of manuscript 2 from data (24) | Ongoing |

June 2014-June 2015 (revised below)

- | | |
|--|--------------|
| 26. Sample collection and 1 year patient follow-ups complete | Not feasible |
| 27. Finalize serum analysis of HHVs for all time points obtained (changes from baseline if detectable) | |

28. Finalize viral DNA PCR for EBV/CMV for all time points obtained (changes from baseline or 4th cycle to long-term follow-up time points if detectable)
29. Finalize IP-10, neopterin cytokine detection for all time-points obtained
30. Flow cytometry assays performed for immune cell changes between patient time-points collected for sero+ individuals: EVB/CMV peptide restimulation
31. Coordinate fatigue data with sero-status, viral-DNA outcomes, inflammatory cytokine status (IP-10, neopterin) and PBMC-virus specific immune activity data
32. Analysis/consultation for statistical significance for measured parameters (30)
33. Prepare, submit 2 manuscripts (manuscripts 3 and 4 from these data)

October 2014-April 2015 (Revised)

Collection of n=14 subject samples, serum & PBMC	Accomplished
CMV, EBV, CRP Serum analysis	Accomplished
CMV, EBV virus-specific T cell stimulation and intracell cytokine measurement	Accomplished
Manuscript submission	Estimated Dec 2015

REFERENCES:

- 1 Pertl, M. M. *et al.* C-reactive protein predicts fatigue independently of depression in breast cancer patients prior to chemotherapy. *Brain, behavior, and immunity* **34**, 108-119, doi:10.1016/j.bbi.2013.07.177 (2013).
- 2 Simanek, A. M. *et al.* Seropositivity to cytomegalovirus, inflammation, all-cause and cardiovascular disease-related mortality in the United States. *PloS one* **6**, e16103, doi:10.1371/journal.pone.0016103 (2011).
- 3 Bennett, J. M. *et al.* Inflammation and reactivation of latent herpesviruses in older adults. *Brain, behavior, and immunity* **26**, 739-746, doi:10.1016/j.bbi.2011.11.007 (2012).
- 4 Li Pira, G. *et al.* A registry of HLA-typed donors for production of virus-specific CD4 and CD8 T lymphocytes for adoptive reconstitution of immune-compromised patients. *Transfusion*, doi:10.1111/trf.12754 (2014).
- 5 Crosswell, A. D., Lockwood, K. G., Ganz, P. A. & Bower, J. E. Low heart rate variability and cancer-related fatigue in breast cancer survivors. *Psychoneuroendocrinology* **45**, 58-66, doi:10.1016/j.psyneuen.2014.03.011 (2014).
- 6 Orre, I. J. *et al.* Higher levels of fatigue are associated with higher CRP levels in disease-free breast cancer survivors. *Journal of psychosomatic research* **71**, 136-141, doi:10.1016/j.jpsychores.2011.04.003 (2011).